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Inactivation of Enteroviruses by Ascorbic Acid and Sodium Bisulfite

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Poliovirus type 1, coxsackievirus type A9, and echovirus type 7 were inactivated by sodium bisulfite and ascorbic acid. Inactivation rates depended upon concentration, temperature, and pH. RNA infectivity was lost during inactivation; the capsid was also altered by these inactivating agents, as determined by enzyme sensitivity assays and by tests of adsorption to cells. Structural modifications of the virus particles were not identical, suggesting that the mechanism of inactivation by ascorbic acid differs from that of sodium bisulfite.

The influence of environmental factors upon the stability of enteroviruses determines the transmissibility of these viruses through food and water. The effects of temperature, pH, food, and food components on the stability of enteroviruses have been reviewed (7). The complexity of foods makes it difficult to predict their suitability as vehicles of virus transmission. Studies with individual solutes provide a more direct indication of their influence on the stability of viruses.

Specific compounds of plant origin have been shown to be antiviral (14). A study on the survival of enteroviruses in retail foods indicated that a food additive, sodium bisulfite, may have caused rapid inactivation of coxsackievirus type B6 in coleslaw (15). In this report we determined the effect of sodium bisulfite on the infectivity of three enteroviruses and on the physical integrity of poliovirus type 1 (Po-1). During this investigation other reducing agents were examined, and the effect of ascorbic acid on enteroviruses was also studied in detail.

MATERIALS AND METHODS

Virus preparations. The origin and preparation of virus stocks in our laboratory of Po-1, strain CHAT, coxsackievirus type A9 (CA-9), strain Bozek, and echovirus type 7 (EC-7) have been described previously (6, 21). The preparation and purification of viruses labeled with [^{32}P]phosphate and [^{14}C]leucine and of unlabeled viruses for experimental use have also been described (21).

Sample preparation and assays. Solutions of L-ascorbic acid (Fisher Scientific Co.), sodium metabisulfite (Allied Chemical), and sodium bisulfite (Mallinckrodt Chemical Works) were prepared with deionized water unless stated otherwise. Virus and viral RNA inactivation were measured by infectivity titrations (plaque-forming units [PFU] per milliliter) as previously described (21). Hemagglutination titrations of EC-7 were done with 0.01 M phosphate-buffered saline (pH 6.2) and human type O erythrocytes in glass tubes (13 by 100 mm). The mixtures were incubated for 1 h at 36°C.

Radioisotope-labeled viruses were used to determine changes in enzyme sensitivity, adsorption to filter membranes, adsorption to cells, and rate of sedimentation of inactivated virions. Precipitation of inactivated virus samples with cold trichloroacetic acid before and after treatment with the enzymes chymotrypsin (500 μg/ml, 22.5 U, alpha-chymotrypsin 3× crystallized, Sigma Chemical Co.) and ribonuclease (RNase; 10 μg/ml, crystalline 5×, Nutritional Biochemicals Corp.) has been described previously (21). Enteroviruses adsorb to cellulose nitrate membranes, but not to cellulose acetate membranes, whose pore sizes greatly exceed the diameter of the virus particles (4). This phenomenon resulted in the development in our laboratory of a rapid method to determine labeling specificity of radioactive enteroviruses (11). We used this filtration method to analyze inactivated virus samples. Samples were diluted in phosphate-buffered saline and then filtered through 0.2-μm-porosity membranes; cellulose nitrate filter membranes were GSWP02500 (Millipore Corp.), and cellulose acetate filter membranes were Metricel GA-8 (Gelman Instrument Co.).

Polioviruses adsorb to cultured primate cells and do not adsorb to nonprimate cells (12). The representative primate cells were HeLa cells from R. R. Rueckert, University of Wisconsin. The nonprimate cells were MDBK cells, a continuous cell line of bovine kidney cells obtained from the American Type Culture Collection. Cells were washed and suspended in Saline Y (71.4 g of NaCl per liter, 2.04 g of KCl per liter, 1.36 g of KH_2PO_4 per liter, and 1.74 g of K_2HPO_4 per liter) containing 0.003% ethylenediaminetetraacetate and 1% calf serum. A 10-ml quantity of the virus-cell suspension was stirred in a 50-ml Erlenmeyer flask for 2 h on a magnetic stirrer at room temperature. The

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amount of radioactivity in the cell-free phase was determined at zero time and at 2 h.

Rate-zonal sedimentation of inactivated virus samples was done in 0.05 M tris(hydroxymethyl)-aminomethane-buffered (pH 7.5) sucrose gradients. A preparative ultracentrifuge (Beckman model L) and swinging bucket rotor types SW25.1 and SW39L were used at the speeds and times stated in the text. Fractions were collected dropwise from the bottom of the tube and assayed for radioactivity.

RESULTS

Loss of infectivity. Solutions containing bisulfite as sodium bisulfite ($NaHSO_3$) or as sodium metabisulfite ($Na_2S_2O_5$) readily inactivated Po-1 at 4, 22, and 30°C. The effect of $Na_2S_2O_5$ on the infectivity of other enteroviruses was then compared with that on Po-1. CA-9, EC-7, and Po-1 were titrated for infectivity after 0, 6, and 24 h at room temperature in various concentrations of $Na_2S_2O_5$. Inactivation increased with time and with $Na_2S_2O_5$ concentration for each of the three enteroviruses (Table 1).

Because bisulfite is generally considered a reducing agent and reducing agents such as dithiothreitol and reduced glutathione have been shown to protect echovirus type 6 from high-temperature (45°C) inactivation (10), we determined the effect of these reducing agents and of ascorbic acid on the infectivity of enteroviruses at moderate temperatures. Neither 0.1 M dithiothreitol nor 0.1 M reduced glutathione affected inactivation of CA-9 at 30°C, but CA-9 was inactivated in ascorbic acid solutions. CA-9 was somewhat more susceptible than Po-1 to inactivation by ascorbic acid (Fig. 1).

Ascorbic acid and bisulfite solutions are acidic. The pH values of 0.01, 0.1, 1.0, and 10 mM $Na_2S_2O_5$ in the experiment reported in Table 1 were 6.0, 5.0, 4.3, and 3.6, respectively. The pH values of 0.01, 0.1, and 1.0 mM ascorbic acid in the experiment shown in Fig. 1 were 6.3, 4.6, and 3.7, respectively. In view of our previous findings (21), the inactivation of enteroviruses in these solutions probably is not primarily an effect of hydrogen ion concentration.

TABLE 1. *Inactivation of enteroviruses by $Na_2S_2O_5$ at room temperature*

Virus	$Na_2S_2O_5$ concn (mM)	Inactivation (log_{10}) at[a]: 6 h	24 h
Po-1	0.01	0.03	0.36
	0.1	0.22	0.96
	1.0	0.98	2.28
	10.0	>3.15	>4.15
CA-9	0.01	0.42	1.14
	0.1	0.81	1.31
	1.0	0.81	1.91
	10.0	2.67	>4.71
EC-7	0.01	0.10	0.36
	0.1	0.50	0.37
	1.0	0.27	1.31
	10.0	2.38	>4.98

[a] Initial virus titer was 7.0 log_{10} PFU/ml.

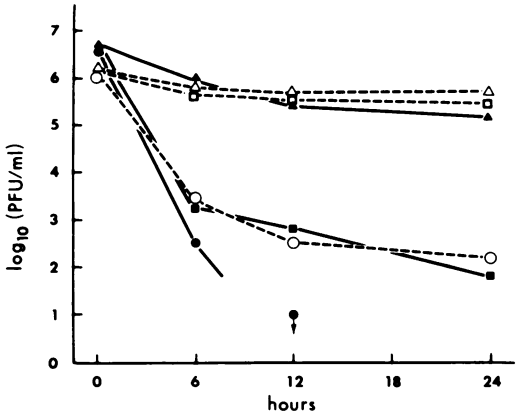

FIG. 1. *Inactivation of enteroviruses by ascorbic acid at 30°C. Symbols: Po-1 in 0.01 (△), 0.1 (□), and 1 (○) mM ascorbic acid solutions and CA-9 in 0.01 (▲), 0.1 (■), and 1 (●) mM ascorbic acid solutions.*

To determine the influence of pH on ascorbic acid inactivation of Po-1, 0.1 M ascorbic acid was added to 0.01 and 0.02 M Na_2HPO_4 to obtain pH values of 7.0 and 5.0. The control samples consisted of citric acid in Na_2HPO_4 solutions. No inactivation of Po-1 occurred in citric acid solutions at pH 7.0 and 5.0, but the inactivation of Po-1 was greater at pH 5.0 than at pH 7.0 in the presence of 11.2 mM ascorbic acid (Table 2). At pH 7.0 there was a greater loss of infectivity of Po-1 in 11.2 mM than in 4.5 mM ascorbic acid. A similar experiment with Po-1 and sodium bisulfite instead of ascorbic acid showed essentially the same effect. Inactivation of enteroviruses in these solutions is evidently dependent on ascorbic acid or sodium bisulfite concentration and, secondarily, upon acidity.

Loss of RNA infectivity and hemagglutinins. If the inactivation process is limited to the protein, the RNA within the protein coat should remain infectious. This can be determined by assaying the virus suspension in the presence of diethylaminoethyl-dextran, one of several substances that facilitate the entry of RNA and inactivated viruses into cells (2). In the reverse situation, RNA infectivity may be lost without a detectable change in the protein coat. For enteroviruses capable of agglutinating erythrocytes, the hemagglutination assay is a convenient method to monitor possible alterations in the protein coat.

TABLE 2. *Influence of pH on the inactivation of Po-1 by ascorbic acid at 30°C*

Anion	Concn[a] (mM)	pH	Inactivation (log_{10}) at:	
			4 h	21 h
Ascorbate	4.5	7.0	1.33	2.19
	11.2	7.0	2.61	3.36
	11.2	5.0	3.87	>5.15
Citrate	1.3	7.0	ND[b]	−0.16[c]
	6.8	5.0	0.20	0.03

[a] The concentration of ascorbate or citrate in the test sample was calculated from the volume of 0.1 M ascorbic acid or 0.1 M citric acid added to 0.01 or 0.02 M Na_2HPO_4 to obtain pH values of 7.0 and 5.0.

[b] ND, Not determined.

[c] Infectivity titer was 0.16 log_{10} PFU/ml greater than at 0 h.

EC-7 was used to determine the effect of sodium bisulfite and ascorbic acid on the RNA and protein coat of enteroviruses. This virus, in 40 mM $NaHSO_3$ at pH 6.0, showed a decrease in infectivity of 1 log_{10} PFU/ml in 20 h and at least 7 log_{10} PFU/ml after 68 h at 22°C (Table 3). The hemagglutination titer had decreased by only eightfold after 68 h. Residual RNA infectivity was not detected either by assaying directly for RNA infectivity or after extracting the RNA with phenol. These results indicate that the RNA infectivity of the whole virus was lost during inactivation in $NaHSO_3$ solutions and that the loss of hemagglutinins occurred after the loss of virus infectivity.

Residual hemagglutinating activity was not found for EC-7 inactivated in ascorbic acid solutions. However, the rate of loss of hemagglutinins was slower than that of virus infectivity. The titer of infectious RNA of EC-7 never exceeded that of the whole virus during inactivation in ascorbic acid solutions, a result similar to inactivation by bisulfite. Although the infectivity of the RNA in EC-7 is destroyed by both chemicals, the inactivation process is probably not the same because of the differential effect on the hemagglutinins.

Physical integrity. To detect other possible alterations to enteroviruses that may occur during inactivation in either ascorbic acid or bisulfite solutions, the effect of these agents on the impermeability of the protein coat of poliovirus was determined. Enteroviruses are generally resistant to nucleases and proteases (5). If the impermeability of the protein coat is altered during inactivation, the RNA within the protein coat may be released or become accessible to RNase. Adsorption to filter membranes can be used to determine whether RNA within virions is released during inactivation. Enteroviruses adsorb to cellulose nitrate membranes whose pore sizes greatly exceed the diameter of virions (4). Single-stranded RNA does not adsorb as efficiently as proteins to cellulose nitrate filter membranes. We found 18% adsorption of phenol-extracted RNA from poliovirus to Millipore filter membranes of 0.22-μm porosity (unpublished data).

Poliovirus was labeled with [^{32}P]phosphate, purified, and then added to 2 mM ascorbic acid. After various times at 30°C, the virus suspension was assayed for infectivity, adsorption to filter membranes, and solubility in trichloroacetic acid before and after RNase treatment. During inactivation, the amount of label precipitated by trichloroacetic acid decreased at a rate similar to the decrease in amount of label that adsorbed to a filter membrane (Fig. 2). This suggests that some hydrolysis and release of poliovirus RNA occurred. The protein coat was also modified during inactivation, because the sensitivity of RNA to RNase increased during inactivation.

Similar experiments were done with labeled Po-1 in $NaHSO_3$ solutions. After a 99.3% reduction of infectivity, 98% of the label remained precipitable by trichloroacetic acid before RNase treatment, and 82% of the label remained precipitable after RNase treatment. The label in this sample adsorbed completely to a Millipore filter membrane, indicating that the RNA remained inside the virion. However, the RNA was sensitive to RNase, indicating that modification of the protein coat had occurred. Controls in our filtration studies were usually passed through Gelman cellulose acetate membranes, which do not adsorb enteroviruses (4); however, during inactivation in $NaHSO_3$ solutions, [^{32}P]-phosphate-labeled Po-1 showed increased adsorption to Gelman membranes (Fig. 3). This

TABLE 3. *Effect of $NaHSO_3$ on the hemagglutinins, RNA infectivity, and whole-virus infectivity of EC-7*[a]

Time (h)	Assay method			
	Hemagglutination (HA units)[b]	Standard (PFU/ml)	RNA[c] (PFU/ml)	Phenol extraction-RNA[d] (PFU/ml)
0	256	2.2×10^8	1.4×10^8	7.0×10^4
20	256	2.5×10^7	7.5×10^6	4.0×10^3
68	32	$<1.0 \times 10^1$	$<2.5 \times 10^1$	$<5.0 \times 10^2$

[a] One part of virus suspension to four parts of 50 mM $NaHSO_3$ (vol/vol) was stored at 22°C and then assayed for infectivity at the indicated times.

[b] Reciprocal of hemagglutination titer.

[c] Virus samples were assayed directly for infectious RNA as described in the text.

[d] Test samples were treated with cold phenol as described in the text and then assayed for infectious RNA.

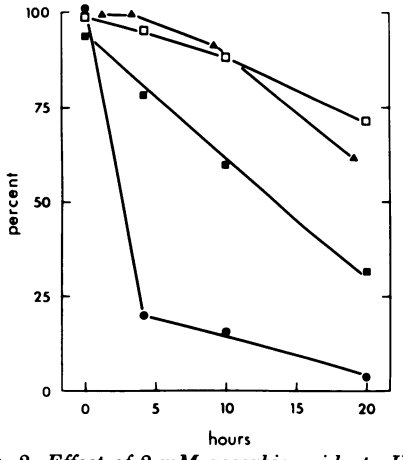

FIG. 2. *Effect of 2 mM ascorbic acid at pH 7 on [^{32}P]phosphate-labeled Po-1 at 30°C. Symbols: ●, infectivity (PFU); □, amount precipitable by trichloroacetic acid (cpm); ■, amount precipitable by trichloroacetic acid after RNase (10 μg/ml) treatment (cpm); ▲, adsorption to Millipore filter membrane (cpm).*

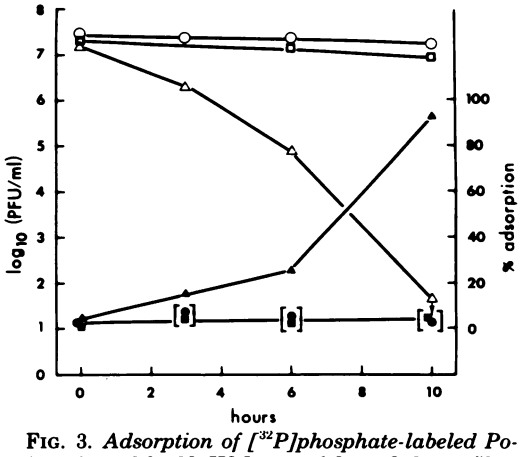

FIG. 3. *Adsorption of [^{32}P]phosphate-labeled Po-1, inactivated by $NaHSO_3$ at 30°C, to Gelman filter membranes. Symbols: infectivity in 0 (○), 2.5 (□), and 25 (△) mM $NaHSO_3$ and adsorption of radioactivity to filter membranes from 0 (●), 2.5 (■), and 25 (▲) mM $NaHSO_3$.*

adsorption was prevented by pretreating the filter membrane with serum, indicating that retention of inactivated virions by Gelman membranes was not due to aggregation. Furthermore, phenol-extracted RNA from poliovirus did not adsorb significantly to Gelman or Millipore filter membranes before or after $NaHSO_3$ treatment. These results indicate that the protein coat of poliovirus was altered during inactivation and that the RNA remained associated with protein.

To ascertain further the relationship between RNA and the protein coat of inactivated virions, samples of inactivated virus were analyzed by centrifugation in sucrose gradients. Poliovirus labeled with [^{32}P]phosphate was inactivated in 2 mM ascorbic acid and then centrifuged in a 10 to 40% sucrose gradient. Approximately 50% of the radioactivity appeared to sediment at a rate similar to that of a control virus preparation (Fig. 4). In fraction 9 (Fig. 4b) the label was precipitated by trichloroacetic acid, but 78% of the label was trichloroacetic acid soluble after RNase treatment. The radioactivity that remained at the top of the sucrose gradient was found to be soluble in trichloroacetic acid. These results resemble those shown in Fig. 2. When [^{32}P]phosphate-labeled Po-1 inactivated by $NaHSO_3$ was subjected to sucrose gradient centrifugation, the label of a sample showing 99.6% inactivation sedimented like that in unaltered virions. No radioactivity appeared at the top of the sucrose gradient. Only 40% of the radioactivity was recovered in these experiments. The amount of label recoverable from sucrose gradients decreased as the proportion of virus inactivated increased. The lost radioactivity could not be recovered by washing the centrifuge tube

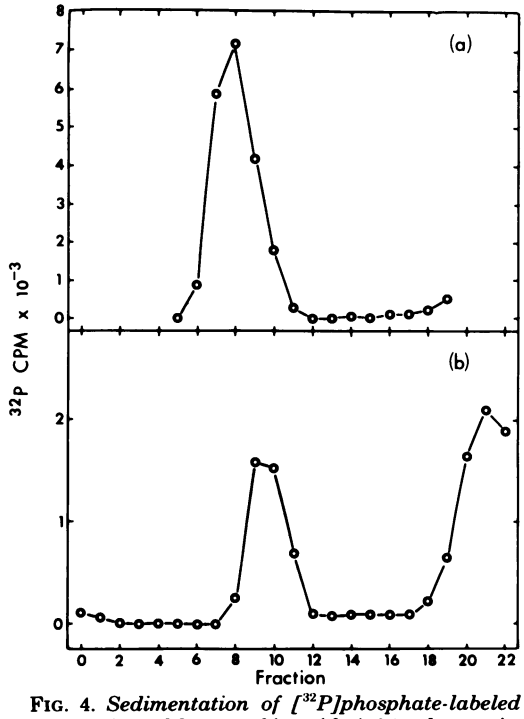

FIG. 4. *Sedimentation of [^{32}P]phosphate-labeled Po-1 inactivated by ascorbic acid. A 0.5-ml quantity of (a) control virus suspension and of (b) treated virus suspension (92% inactivation) were layered on a 4.5-ml 10 to 40% sucrose gradient. After 3 h at 30,000 rpm in an SW39L rotor, fractions were collected from the bottom of the tube and assayed for radioactivity.*

with a phosphate buffer, but was partially recovered by washing with a radioisotope-decontaminating detergent (Isoclean, Amersham/Searle). These losses of label were apparently due to adsorption and occurred in various plastic centrifuge tubes and glass tubes. Filtration through Gelman membranes had also shown nonspecific adsorption (Fig. 3).

The coat protein of enteroviruses is denatured during high-temperature inactivation; this offered a basis for comparison with the effects of ascorbic acid and $NaHSO_3$. Inactivation of [^{14}C]leucine-labeled Po-1 by heat, ascorbic acid, and $NaHSO_3$ resulted in an increased sensitivity to chymotrypsin (Table 4). The ascorbic acid-inactivated virions were the most sensitive and were the only ones that showed trichloroacetic acid-soluble protein without chymotrypsin treatment. A decrease in adsorption to cells occurred as a result of each inactivation process. This could have been due to loss or denaturation of the polypeptide VP 4, which is necessary for virus adsorption to cells (8). A consistent finding was the increased adsorption of inactivated virions to nonprimate MDBK cells.

The inactivation of Po-1 by ascorbic acid and $NaHSO_3$ described above was stopped by freezing the samples. These were then thawed and layered on sucrose gradients to determine the sedimentation rate of the inactivated particles. EC-7 was included in each gradient as a marker of unaltered virions. The ascorbic acid-treated sample, 94% inactivated, showed two peaks by rate sedimentation (Fig. 5). Fractions 9 and 10 contained the marker of unaltered virions (EC-7) and one band of inactivated virions. A second band sedimented at a rate about one half that of unaltered virions.

Most of the radioactivity in the frozen sample of Po-1, 96.3% inactivated by $NaHSO_3$, was lost before and during centrifugation. The remaining radioactivity was evenly distributed from the fraction containing the marker of unaltered virions (EC-7) to the top of the gradient. This differed from the result of sucrose gradient centrifugation of [^{32}P]phosphate-labeled Po-1 inactivated by $NaHSO_3$, which showed a sedimentation rate similar to that of unaltered virions. The lack of a discernible peak of radioactivity in this sample was probably due to freezing and

TABLE 4. *Modification of [^{14}C]leucine-labeled Po-1 by heat, ascorbic acid, and $NaHSO_3$*[a]

Sample	Inactivation (%)	Adsorption (%) of label to cells in suspension[b]		Solubility (%) of label in trichloroacetic acid after:	
		MDBK	HeLa	No enzyme	Chymotrypsin
Control	0	0	39	2	3
Heated	99.4	6	4	0	17
Ascorbic	94.0	3	16	2	34
acid	99.9	ND[c]	ND	28	51
$NaHSO_3$	96.3	8	11	8	30
	99.9	ND	ND	5	23

[a] Virus was suspended in phosphate-buffered saline and heated for 5 min at 55°C or was inactivated at 30°C by 4 mM ascorbic acid in 0.067 M phosphate buffer (pH 7.0) or by 20 mM $NaHSO_3$.

[b] The cell suspensions consisted of 10 ml of 1.2×10^7 cells per ml and 2.3×10^9, 1.4×10^7, 1.4×10^8, and 8.5×10^7 PFU for the control, heated, ascorbic acid, and $NaHSO_3$ samples, respectively.

[c] ND, Not determined.

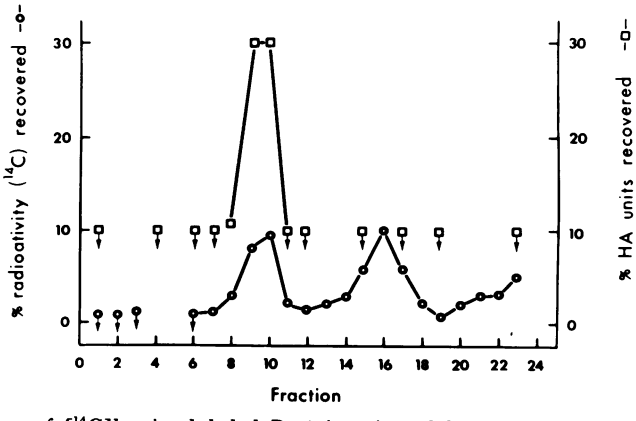

FIG. 5. *Sedimentation of [^{14}C]leucine-labeled Po-1 inactivated by 4 mM ascorbic acid at 30°C. After inactivation, the virus suspension was mixed with sucrose (final concentration, 5%) and EC-7, which was added as a control of unaltered virions. A 1.6-ml quantity of this virus suspension was layered on 32 ml of 15 to 40% sucrose in tris(hydroxymethyl)aminomethane buffer and centrifuged for 4.5 h at 24,000 rpm with an SW25.1 rotor. Fractions were collected from the bottom of the tube and assayed for radioactivity (Po-1) and hemagglutinins (EC-7).*

thawing, which resulted in disruption of the inactivated virions.

DISCUSSION

Sodium bisulfite inactivated three enteroviruses, Po-1, CA-9, and EC-7, with equal efficiency. Inactivation of enteroviruses occurred at 4, 22, and 30°C with sodium bisulfite ($NaHSO_3$) or sodium metabisulfite ($NA_2S_2O_5$). Inactivation was slightly more rapid in $Na_2S_2O_5$ than in an equal molar solution of $NaHSO_3$ (data not shown). Our results support the suggestion by Lynt (15) that the presence of sodium bisulfite in coleslaw may have caused a significant reduction of infectivity of coxsackievirus type B6.

Because bisulfite is considered a moderately strong reducing agent, other reducing agents were tested for effect on the infectivity of enteroviruses. Although dithiothreitol and reduced glutathione have been shown to protect echovirus type 6 from high-temperature (45°C) inactivation (10), neither of these agents caused inactivation or protected the infectivity of CA-9. Another reducing agent, ascorbic acid, did inactivate CA-9. This virus was more susceptible than Po-1 to inactivation by ascorbic acid. Poliovirus was shown to be inactivated by ascorbic acid over 40 years ago (13). Several other compounds of plant origin in addition to ascorbic acid inactivate poliovirus (14). A number of DNA- and RNA-containing bacteriophages are also inactivated by ascorbic acid and other reducing agents (17–19). Although the extracellular particles of rhinovirus are not inactivated by ascorbic acid, the replication of this virus is suppressed in the presence of ascorbic acid (22).

Several factors affect the rate of inactivation of enteroviruses by ascorbic acid and sodium bisulfite. Serum decreases the rate of inactivation (data not shown). The inactivation rate increases with temperature; however, our studies did not include the high temperatures at which reducing agents protected echovirus type 6 against thermal inactivation (10). The pH value of ascorbic acid and bisulfite solutions influenced the rate of inactivation of Po-1; inactivation was greater at pH 5 than at pH 7. This effect is not due primarily to an increased concentration of hydrogen ions, because the rate of inactivation of Po-1 in the absence of ascorbic acid and bisulfite is essentially the same within the pH range of 4 to 7 (21).

Only certain ion forms of ascorbic acid and sodium bisulfite may be active against enteroviruses. Murata and Kitagawa (16) have shown that the inactivation of a double-stranded DNA bacteriophage by ascorbic acid is extremely rapid below pH 6.2 or above pH 8.5 and that ascorbic acid is more effective in tris(hydroxymethyl)aminomethane buffer than in phosphate buffer. Air, oxidizing agents, and transition metal ions enhanced the rate of inactivation of phage by ascorbic acid, whereas nitrogen, other reducing agents, and radical scavengers prevented inactivation (16). They concluded that the inactivating effect of ascorbic acid is oxygen dependent and is caused by free radicals formed during the autoxidation of ascorbic acid. In one experiment, we found that the inactivation of CA-9 in ascorbic acid was enhanced when $CuSO_4$ was present and reduced in the presence of ethylenediaminetetraacetate (data not shown). However, the differences that we observed were small compared with those reported by Murata and Kitagawa. More studies are needed to identify the active forms of ascorbic acid and sodium bisulfite and the optimum conditions for their inactivation of enteroviruses.

Virus particles inactivated by ascorbic acid and by sodium bisulfite show some similarities. The modes of action of ascorbic acid and sodium bisulfite are probably something other than reduction of disulfide bonds, inasmuch as the other reducing agents that were tested were ineffective against CA-9. When EC-7 was inactivated by these agents, infectivity of the RNA was lost along with that of the whole particle. The nucleic acid of inactivated particles was accessible to RNase, and the protein coat was sensitive to chymotrypsin. Po-1, inactivated by ascorbic acid or sodium bisulfite, lost its ability to adsorb to what would otherwise have been its host cells.

Virions inactivated by ascorbic acid also show differences from those inactivated by sodium bisulfite. Infectivity was lost more rapidly than hemagglutinin titer when EC-7 was inactivated by ascorbic acid, but residual infectivity was still present when hemagglutinins could no longer be detected. Inactivation of Po-1 by ascorbic acid, but not by sodium bisulfite, yielded trichloroacetic acid-soluble derivatives of ^{14}C-labeled protein and [^{32}P]phosphate-labeled nucleic acid. Po-1 labeled with [^{32}P]phosphate or [^{14}C]leucine was examined further by sucrose density gradient centrifugation after inactivation. Some particles inactivated by ascorbic acid sedimented at the same rate as unaltered virions. These noninfectious particles were precipitable by trichloroacetic acid, but their nucleic acid was sensitive to RNase. Inactivation by ascorbic acid also results in some breakdown of virus particles; some ^{14}C-labeled material sedimented at about 80S, and some remained near the meniscus of the sucrose gradient. Some ^{32}P-labeled material also remained near the meniscus; this was hydrolyzed RNA, as shown by its trichloroacetic acid solubility.

Sodium bisulfite also had some distinctive effects upon these enteroviruses. EC-7 inactivated by sodium bisulfite showed a slight loss of hemagglutinins after complete loss of infectivity; rather than being destroyed, the hemagglutinins may be lost because the inactivated particles adsorb to the test tube walls. Similarly, Po-1 inactivated by sodium bisulfite was difficult to characterize by sucrose gradient centrifugation because the inactivated particles adsorbed nonspecifically to the centrifuge tubes and to other surfaces. Inactivated, ^{32}P-labeled particles showed an unaltered sedimentation rate, whereas inactivated, ^{14}C-labeled virus contained a mixture of particles with sedimentation coefficients ranging downward from 160S, perhaps because freezing and thawing disrupted the inactivated virions in this sample. The amount of material lost due to adsorption prevents a complete description of the nature of the inactivated virion. Nonspecific adsorption has been reported with other picornaviruses inactivated in other ways. Degradation products of mouse Elberfeld virus were not completely recovered after centrifugation in glycerol gradients; the 14S subunits were recovered in near-quantitative yields, but the 5S subunits showed 70% or more adsorption to container surfaces (9). During inactivation of rhinovirus type 2, the polypeptide VP 4 was released and readily adsorbed to test tube walls; the authors of the report quoted M. Breindl as having observed a similar occurrence with poliovirus (20).

The physical integrity of the Po-1 virion is more subtly affected by sodium bisulfite than by ascorbic acid. Bisulfite treatment of bacteriophage MS 2 produced covalent cross-linkages between the RNA and the protein coat (3). Treatment of an RNA-containing plant virus, turnip yellow mosaic virus, with bisulfite reduced the cytosine content of the RNA to about 26% without appreciably affecting the protein coat (1). Our results suggest that the nucleic acids of enteroviruses are the primary target of bisulfite. Because the loss of hemagglutinins and the increase of nonspecific adsorption of enteroviruses occur after the loss of infectivity, these alterations to the protein coat are probably secondary effects. The double-stranded DNA of bacteriophage J 1 appears to be the target of ascorbic acid; inactivated phage did not show a loss of ability to adsorb to the host bacterial cell, but ascorbic acid-treated phage DNA did show single-strand breaks in the DNA molecule (16). The physical integrity of ascorbic acid-treated enteroviruses is significantly altered. However, our results do not indicate clearly whether the primary event of inactivation occurs in the nucleic acid or at the surface of the virion. Our results do show that ascorbic acid and bisulfite may inactivate enteroviruses during transmission from one host to another and that, if inactivation occurs, the effect on the virus would be irreversible.

ACKNOWLEDGMENTS

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